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Selective blockade of metabotropic glutamate receptor subtype 5 is neuroprotective

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Abstract

We have used potent and selective non-competitive antagonists of metabotropic glutamate receptor subtype 5 (mGlu5) —- 2-methyl-6-phenylethynylpyridine (MPEP), [6-methyl-2-(phenylazo)-3-pyridinol] (SIB-1757) and [(*E*)-2-methyl-6-(2-phenylethenyl)pyridine] (SIB-1893) — to examine whether endogenous activation of this particular metabotropic glutamate receptor subtype contributes to neuronal degeneration. In cortical cultures challenged with *N*-methyl-D-aspartate (NMDA), all three mGlu5 receptor antagonists were neuroprotective. The effect of MPEP was highly specific because the close analogue, 3-methyl-6-phenylethynylpyridine (iso-MPEP), which did not antagonize heterologously expressed mGlu5 receptors, was devoid of activity on NMDA toxicity. Neuroprotection by mGlu5 receptor antagonists was also observed in cortical cultures challenged with a toxic concentration of β-amyloid peptide. We have also examined the effect of mGlu5 receptor antagonists in in vivo models of excitotoxic degeneration. MPEP and SIB-1893 were neuroprotective against neuronal damage induced by intrastriatal injection of NMDA or quinolinic acid. These results indicate that mGlu5 receptors represent a suitable target for novel neuroprotective agents of potential application in neurodegenerative disorders.

Keywords: Excitotoxicity; β-Amyloid toxicity; Neuroprotection; mGlu5 receptors; Non-competitive antagonists; MPEP; SIB-1757; SIB-1893

1. Introduction

Although the role of metabotropic glutamate receptor subtypes 1 and 5 (mGlu1 and mGlu5) in neurodegeneration is still controversial, there is general consensus on the neuroprotective activity of group-I mGlu receptor antagonists (reviewed by Nicoletti et al., 1999). Drugs that selectively antagonize mGlu1 receptors [2-methyl-4-carboxyphenylglycine (LY367385), (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) and 4-carboxyphenylglycine] or behave as mixed mGlu1 and mGlu5 receptor antagonists (such as LY367366, α-thioxanthyl-9-methyl analogue of (*S*)-4-carboxyphenylglycine) attenuate excitotoxic neuronal death both in vitro and in vivo and protect vulnerable neurons against ischemic damage (Bruno et al., 1999; Strasser et al., 1998; Peruginelli et al., 1999, Pellegrini-Giampietro et al., 1999). However, the contribution of mGlu5 receptor blockade to neuroprotection is unknown at present. We have addressed this issue by using the novel, highly selective mGlu5 receptor antagonists, 2-methyl-6-phenylethynylpyridine (MPEP), [6-methyl-2-(phenylazo)-3-pyridinol] (SIB-1757) and [(*E*)-2-methyl-6-(2-phenylethenyl)pyridine] (SIB-1893) (Varney et al., 1999a,b; Gasparini et al., 1999). These drugs do not interact with the glutamate-recognition site located on the amino-terminal domain of mGlu5 receptors, and therefore behave as non-competitive antagonists (Pagano et al., 1999). This makes MPEP, SIB-1757 and SIB-1893 insensitive to the ambient concentrations of glutamate and, therefore, good tools for examination of the role of mGlu5 receptors in neurodegeneration.

We now report that selective blockade of mGlu5

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receptors potently protects cultured cortical neurons against *N*-methyl-D-aspartate (NMDA) or β-amyloid toxicity, and is also protective against excitotoxic neurodegeneration in in vivo models.

2. Methods

2.1. Assessment of mGlu5 receptor activation in a heterologous expression system

Stimulation of polyphosphoinositide (PI) hydrolysis by quisqualate was assessed in Ltk^- cells expressing human mGlu5a receptors, as described by Litschig et al. (1999). MPEP or 3-methyl-6-phenylethynylpyridine (iso-MPEP) were applied to the incubation buffer immediately prior to quisqualate.

2.2. Mixed cortical cultures

Mixed cortical cell cultures containing both neurons and astrocytes were prepared from fetal mice at 14–16 days of gestation, as described previously (Rose et al., 1992). Briefly, dissociated cortical cells were plated in 15 mm multiwell vessels (Falcon Primaria, Lincoln Park, NJ) on a layer of confluent astrocytes (7–14 days in vitro), using a plating medium of Modified Eagle's Medium (MEM)–Eagle's salts (supplied glutamine-free) supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, glutamine (2 mM) and glucose (final concentration 21 mM). Cultures were kept at 37°C in a humified 5% CO_2 atmosphere. After 3–5 days in vitro, non-neuronal cell division was halted by 1–3 days' exposure to 10 μM cytosine arabinoside, and cultures were shifted to a maintenance medium identical to the plating medium but lacking fetal serum. Subsequent partial medium replacement was carried out twice a week. Only mature cultures (13–14 days in vitro) were used for the experiments.

2.3. Glial cell cultures

Glial cell cultures were prepared as described previously (Rose et al., 1992) from postnatal mice (1–3 days after birth). Dissociated cortical cells were grown in 15 mm multiwell vessels (Falcon Primaria) using a plating medium of MEM–Eagle's salts supplemented with 10% fetal bovine serum, 10% horse serum, glutamine (2 mM) and glucose (final concentration 21 mM). Cultures were kept at 37°C in a humified CO_2 atmosphere until they reached confluence (13–14 days in vitro). Confluent cultures were then used for the experiments or as a support for mixed cultures.

2.4. Exposure to excitatory amino acids and β-amyloid peptide (β-AP)

Brief exposure to 60 μM NMDA (10 min), in the presence or absence of group-I mGlu receptor antagonists, was carried out in mixed cortical cultures at room temperature in a (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid](HEPES)-buffered salt solution containing (in mM): 120 NaCl, 5.4 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 20 HEPES, 15 glucose. After 10 min the drugs were washed out, and cultures were incubated at 37°C for the following 24 h in medium stock (MS) (MEM–Eagle's supplemented with 15.8 mM $NaHCO_3$ and glucose <25 mM). The toxic fragment of β-amyloid peptide (β-$AP_{(25–35)}$) was solubilized in sterile water at an initial concentration of 2.5 mM and was stored frozen at −20°C for at least 1 week prior to use. Neurodegeneration induced by β-AP (25 μM) for 24–48 h was done in the presence of 10 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX) and 10 μM dizocilpine (MK-801) as described by Copani et al. (1995).

2.5. Assessment of in vitro neuronal injury

Neuronal injury was estimated in all experiments by examination of cultures with phase-contrast microscopy at 100–400×, 1 day after the insult, when the process of cell death was largely complete. Neuronal damage was assessed quantitatively in all experiments by estimation of dead neurons by trypan-blue staining. Stained neurons were counted from three random fields per well.

2.6. Animal preparation

Male Sprague-Dowley rats weighing 250–300 g were used for all the experiments. Rats were injected, under pentothal (50 mg/kg, intraperitoneally) anesthesia, with NMDA (100 nmol/0.5 μl) or NMDA (100 nmol/0.5 μl)+MPEP (5 nmol/0.5 μl) in a stereotaxic frame with nose bar positioned at +5 mm. The sites of injection were the left anterior striatum with the coordinates: 1.5–2.5 mm anterior to the bregma, 2.6 mm lateral from the midline, 5 mm ventral from the surface of the dura, according the atlas of Pellegrino et al. (1992). The injection was made in two different sites, 1 mm antero-posterior apart. Additional groups of animals were injected with quinolinic acid (200 nmol) or quinolinic acid+SIB-1893 (100 nmol) into the caudate nucleus, as described by Sauer et al. (1992). After surgery, rats were housed in separated cages in a temperature-controlled environment on a 12 h light–dark cycle, with free access to water and food. The animals were allowed 7 days to develop an excitotoxic striatal neuronal death induced by NMDA.

2.7. Assessment of in vivo neuronal injury

In animals injected with NMDA, neuronal injury was assessed by performing both histological analysis and measurements of striatal glutamate decarboxylase (GAD) activity. For histological examination, serial frontal sections including the whole caudate nucleus were Nissl-stained and examined by phase-contrast microscopy. For measurements of GAD activity, both striata were dissected and homogenized in 5 mM imidazole buffer containing 0.2% Triton X-100 and 0.1 mM dithiothreitol. One hundred μl of the homogenate were incubated in 10 mM phosphate buffer, pH 7.0, containing 10 mM 2-mercapthoethanol and 0.02 mM pyridoxalphosphate, in the presence of [^{3}H]-glutamic acid (1 μCi; specific activity, 46 Ci/mmol) for 1 h at 37°C; the reaction was stopped by adding 15 μl of 11.8 N $HClO_4$ in ice. Samples were centrifuged and supernatants were injected into a high-performance liquid chromatograph to separate [^{3}H]-γ-aminobutyric acid ([^{3}H]-GABA). The peak corresponding to [^{3}H]-GABA was collected and radioactivity was measured by scintillation spectometry. Proteins were measured by using a commercially available kit (BIO-RAD protein assay, BIO-RAD Laboratories, Munchen, Germany). In animals injected with quinolinic acid or quinolinic acid+SIB-1893, the size of the striatal lesion was assessed by magnetic resonance imaging performed as described previously (Sauer et al., 1992).

2.8. Materials

Tissue culture reagents were from Sigma (Milano, Italy). NMDA, DNQX and MK-801 were purchased from Tocris (Bristol, UK). SIB-1757, SIB-1893, MPEP and its analogue iso-MPEP were synthesized at Novartis Pharma AG (see Gasparini et al., 1999). β-$AP_{(25–35)}$ was obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland).

3. Results

The chemical structures of SIB-1757, SIB-1893, MPEP and iso-MPEP are shown in Fig. 1. The selectivity of MPEP as an antagonist for mGlu5 receptors is shown in Table 1. Note that the drug fails to interact with other mGlu receptor subtypes, ionotropic glutamate receptors or endogenous $P2Y_2$ adenosine triphosphate (ATP) receptors in recombinant cells (for further details, see Gasparini et al., 1999). As opposed to MPEP, iso-MPEP did not antagonize heterologously expressed mGlu5 receptors up to 100 μM (Fig. 2). SIB-1757 and SIB-1893 are known to antagonize recombinant mGlu5 receptors with half-maximal inhibitory concentration (IC_{50}) values of 0.37 μM and 0.29 μM, respectively, and

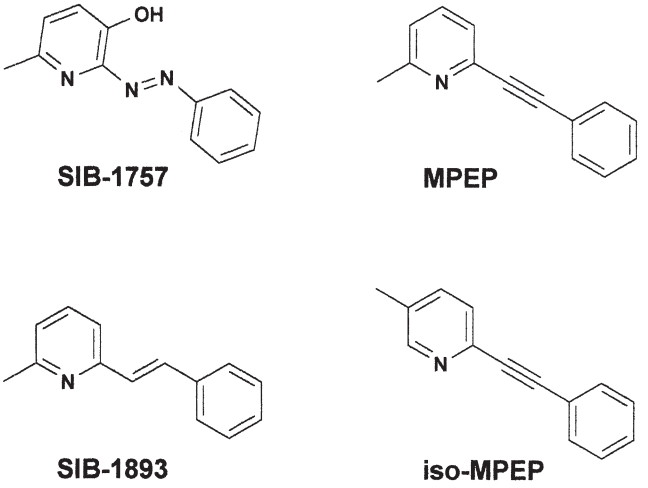

Fig. 1. Chemical structures of SIB-1757, SIB-1893, MPEP and iso-MPEP.

both fail to interact with other mGlu receptor subtypes or with ionotropic glutamate receptors up to 30 μM (Varney et al., 1999a).

To examine the effect of selective mGlu5 receptor blockade on in vitro neuronal degeneration we have used mixed cultures of mouse cortical cells, where mGlu5 receptors are highly expressed and group-I mGlu receptor agonists facilitate excitotoxic death (Bruno et al., 1995). Cultures were transiently exposed to NMDA at concentrations of 60 μM which, in this particular set of experiments, killed about 50–60% of the neuronal population. When MPEP, SIB-1757 or SIB-1893 was coapplied with NMDA they were all neuroprotective in a concentration-dependent fashion. MPEP was the most potent neuroprotectant, in line with its greater potency as an mGlu5 receptor antagonist. In addition, MPEP and SIB-1893 showed approximately the same efficacy, whereas SIB-1757, although more potent than SIB-1893, was less efficacious (Fig. 3). As opposed to MPEP, the inactive analogue iso-MPEP did not substantially affect NMDA toxicity (Table 2).

Cultured cortical neurons were also challenged for 24 h with 25 μM of the toxic fragment of β-amyloid peptide (β-$AP_{(25–35)}$) in the presence of a cocktail of ionotropic glutamate receptor antagonists (10 μM MK-801+30 μM DNQX). Under these conditions, cortical neurons are known to die by apoptosis (Copani et al., 1995). However, due to the lack of phagocytes and the occurrence of secondary necrosis, we could also assess neuronal toxicity by trypan-blue staining. MPEP applied during the 24 h of exposure to β-$AP_{(25–35)}$ showed a high potency in this paradigm of toxicity, and was substantially neuroprotective at concentrations as low as 10 nM. The efficacy of MPEP was comparable to that shown by the group-III mGlu receptor agonist, L-2-amino-4-phosphonobutanoate (L-AP4), used as a reference compound (Copani et al., 1995). SIB-1757 and SIB-1893 were also

Table 1
Selectivity of MPEP for mGlu5 receptors in heterologous expression systems[a] (from Gasparini et al., 1999)

Assay	Receptor	Agonist activity	Antagonist activity
PI hydrolysis	hmGlu1b	None (0.1–100)	None (0.1–100)
	hmGlu5a	None (0.1–100)	IC_{50}=0.032
	P2Y	None (0.1–100)	None (0.1–100)
cAMP	hmGlu2	None (100)	None (100)
	hmGlu4a	None (100)	None (100)
	hmGlu6	20% (100), 0% (10)	None (100)
	hmGlu7b	None (100)	None (100)
	hmGlu8a	None (100)	None (100)
[^{35}S]-GTPγS binding	hmGlu3	None (100)	None (100)
Inward current	hNMDA1A/2A	None (10, 100)	None (10, 100)
	hNMDA1A/2B	None (10, 100)	22% (100), 0% (10)
	ratAMPA(Glu3-flop)	None (10, 100)	None (10, 100)

[a] Human (h) mGlu5a receptors were recombinantly expressed in Ltk$^-$ cells. All other metabotropic receptors were expressed in Chinese hamster ovary (CHO) cells. P2Y ATP receptors are endogenously expressed by CHO cells. Ionotropic receptor activities were recorded in *Xenopus* oocytes (for further details, see Gasparini et al., 1999).

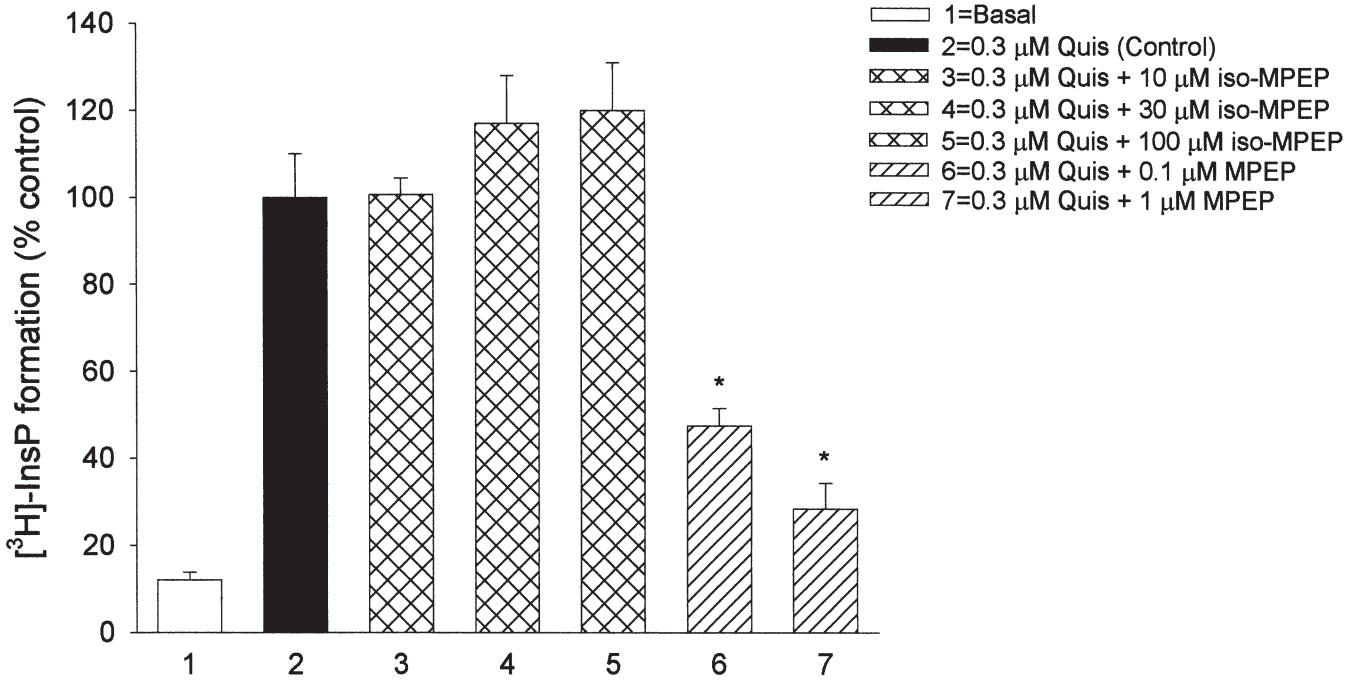

Fig. 2. Stimulation of PI hydrolysis by 0.3 μM quisqualate (Quis) in Ltk$^-$ cells expressing hmGlu5a receptors incubated in the presence of MPEP or iso-MPEP at the concentrations indicated. Values are means±standard error of the mean (SEM) of three to nine determinations from one to three independent experiments. * indicates $P<0.05$ versus Quis alone [one-way analysis of variance (ANOVA)+Fisher PLSD].

neuroprotective against β-$AP_{(25–35)}$ toxicity, although they were less efficacious than MPEP (Fig. 4).

We also examined the effect of mGlu5 receptor antagonists in in vivo models of excitotoxic neuronal death. In rats infused with NMDA (2×100 nmol/0.5 μl), coadministration of MPEP (5 nmol) was neuroprotective, as shown by histological analysis [Fig. 5(A)] or by measurements of GAD activity as an indication of the survival of striatal GABAergic neurons [Fig. 5(B)]. In another set of experiments, we examined the effect of SIB-1893 on striatal toxicity induced by local infusion of quinolinic acid, an endogenous excitotoxin known to activate NMDA receptors (Schwarcz et al., 1983; Ganong and Cotman, 1986). In spite of the large lesion induced by 200 nmol of quinolinic acid, coadministration of SIB-1893 (100 nmol) reduced the lesion volume dramatically (Fig. 6).

4. Discussion

Although mGlu1 and mGlu5 were the first mGlu receptors to be cloned (reviewed in Nakanishi, 1994),

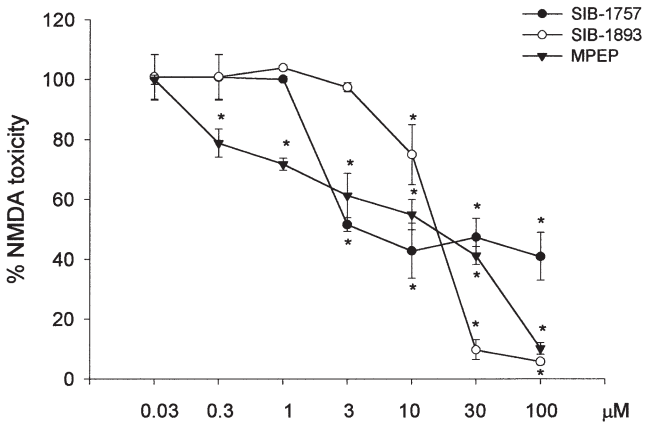

Fig. 3. Concentration-dependent neuroprotection by SIB-1757, SIB-1893 and MPEP against NMDA toxicity in mixed cultures of cortical cells. Values are means±SEM of three to 15 determinations from one to five independent experiments. * indicates $P<0.05$ (one-way ANOVA+Fisher probability at least significant difference (PLSD)).

Table 2
Iso-MPEP, a close analogue of MPEP, fails to affect neuronal degeneration induced by NMDA in cortical cultures[a]

	% NMDA toxicity
NMDA, 60 μM	100±2.5
+MPEP, 30 μM	37±8.2*
+iso-MPEP, 30 μM	102±4.2

[a] Values are mean±SEM of two individual determinations performed in quadruplicate. * indicates $P<0.05$ (one-way ANOVA+Fisher PLSD), if compared with NMDA alone. Neither MPEP nor iso-MPEP had any effect by itself on neuronal viability.

their study has been limited for several years by the lack of selective antagonists. Some phenylglycine derivatives, such as α-methyl-4-carboxyphenylglycine, 4-carboxyphenylglycine and 4-carboxy-3-hydroxyphenylglycine, behave as group-I mGlu receptor antagonists (preferentially as mGlu1a receptor antagonists), but they also interact with group-II mGlu receptors (reviewed by Schoepp et al., 1999). More recently, 2-methyl-4-carboxyphenylglycine (LY367385), (*RS*)-1-aminoindan-1,5-dicarboxylic acid (AIDA) and 7-hydroxyiminocyclopropan[b]chromen-1α-carboxylic acid ethyl ester (CPCCOEt) have been introduced as selective mGlu1 receptor antagonists (Clark et al., 1997; Pellicciari et al., 1995; Moroni et al., 1997; Annoura et al., 1996). LY367366, the α-thioxanthyl-9-methyl analogue of (*S*)-4-carboxyphenylglycine, antagonizes mGlu5 receptors at low micromolar concentrations, but is equally potent in antagonizing mGlu1 receptors and can also recruit group-II and group-III mGlu receptors at concentrations >10 μM (Clark et al., 1998; Thomas et al., 1998). High throughout screening led to the discovery of SIB-1757 and SIB-1893, two phenylpyridine derivatives that antagonize mGlu5 receptors in the low micromolar range (IC_{50}=0.37 and 0.29 μM, respectively) but are highly

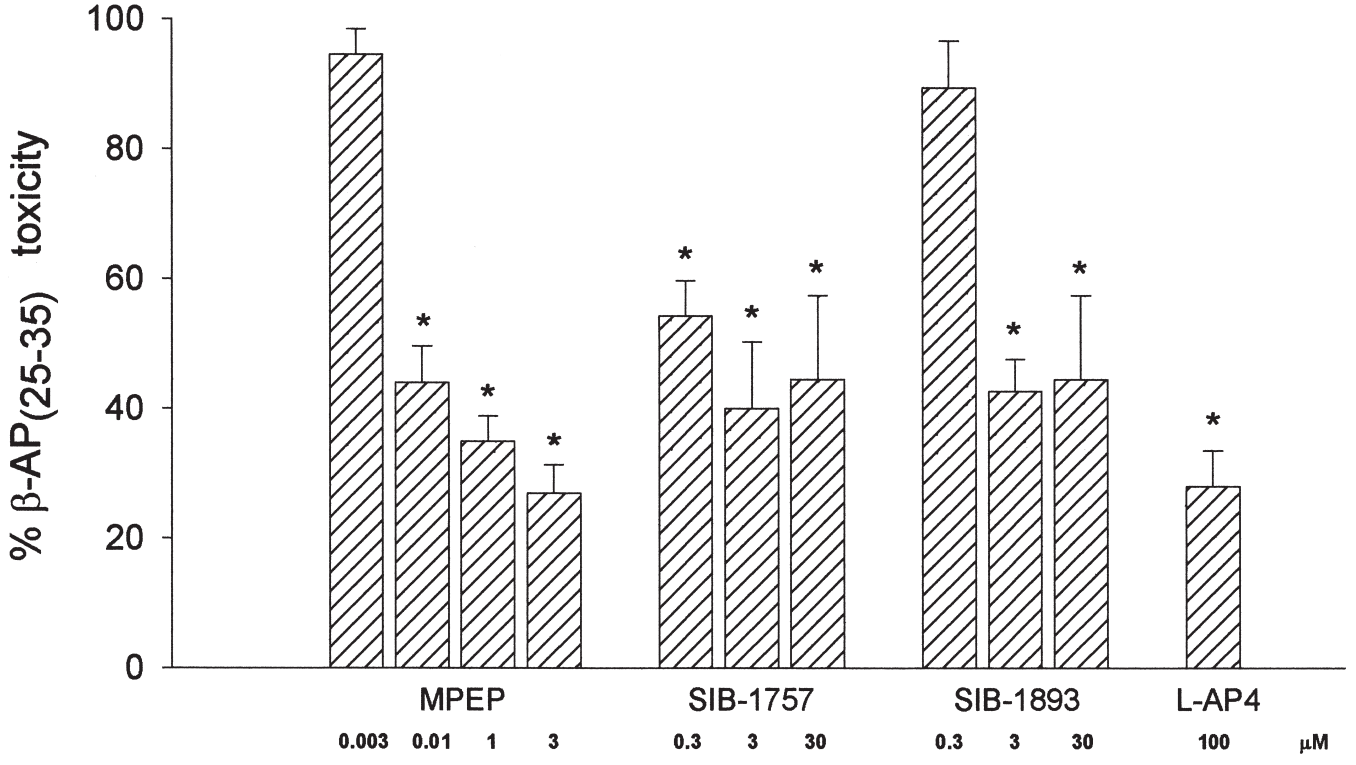

Fig. 4. mGlu receptor antagonists protects cultured cortical neurons against β-amyloid toxicity. Protection by L-AP4 is also shown. Values are means±SEM of six determinations from two independent experiments. * indicates $P<0.05$ versus β-AP alone (one-way ANOVA+Fisher PLSD).

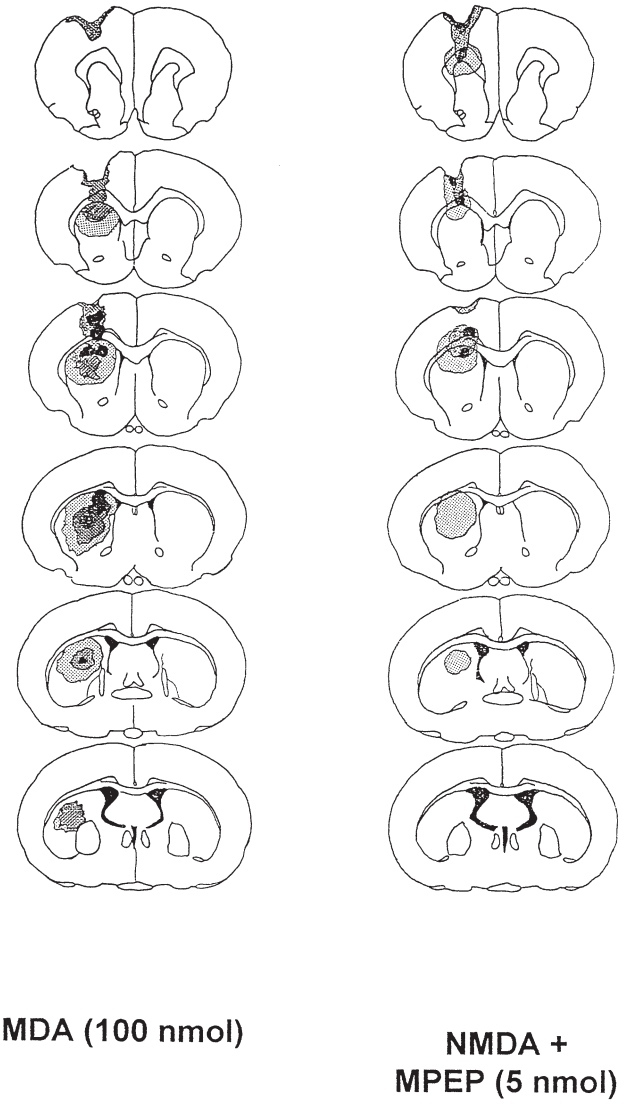

Fig. 5. (A) Serial frontal sections across the extension of the caudate nucleus from a representative animal locally infused with NMDA (100 nmol/0.5 μl×2) or NMDA+MPEP (5 nmol). Necrotic areas are in black, whereas the grey shadow represents the surrounding oedema. (B) MPEP attenuates the loss of striatal GABAergic neurons (expressed as % reduction in GAD activity for each sample versus the contralateral uninjected site) induced by local infusion of NMDA. Values are means±SEM of five determinations. * indicates $P<0.05$ versus NMDA alone (Student's *t*-test).

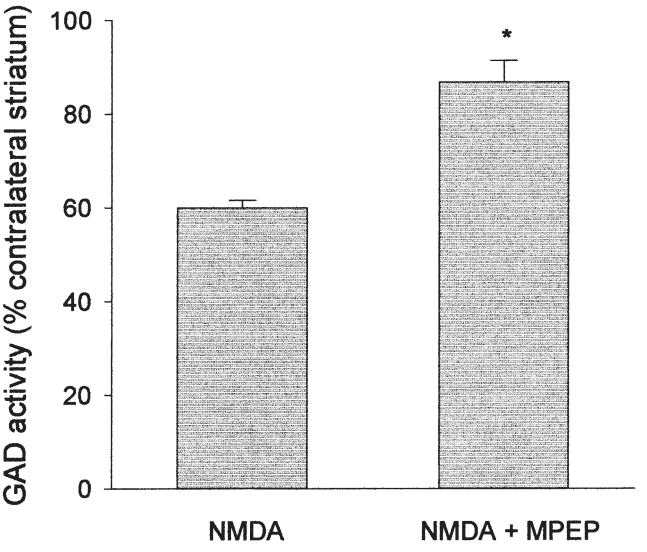

Fig. 5. (*continued*)

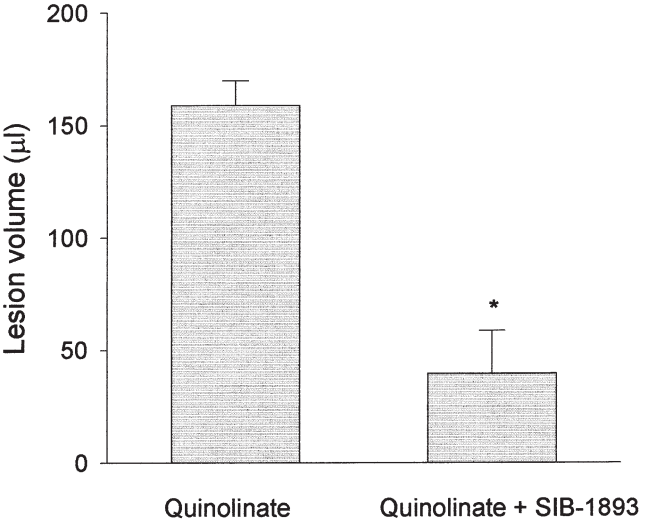

Fig. 6. Protective activity of SIB-1893 (100 nmol) against toxicity induced by local infusion of quinolinic acid (200 nmol/0.5 μl). Lesion size was quantitated by magnetic resonance imaging. * indicates $P<0.05$ versus quinolinic acid alone (Student's *t*-test).

selective with respect to other mGlu receptor subtypes or ionotropic glutamate receptors (Varney et al., 1999a,b). MPEP differs from SIB-1893 in the presence of an ethynyl moiety between the phenyl and pyridine groups, and exhibits a greater potency as an mGlu5 receptor antagonist without losing the selectivity of the SIB compounds (Gasparini et al., 1999). The lack of an α-amino-acidic moiety and a distal acidic moiety in the structures of MPEP, SIB-1757 or SIB-1893 does not allow them to interact with the glutamate-recognition site located on the amino-terminal domain of mGlu5 receptors (Varney et al., 1999a,b; Pagano et al., 1999). At least MPEP interacts with the transmembrane (TM) domains III and VII of mGlu5 receptors, and its binding requires the amino-acid residues Pro655 and Ser658 in TMIII and Ala810 in TMVII of the hmGlu5a receptor (Pagano et al., 1999).

All of these non-competitive mGlu5 receptor antagonists were neuroprotective against excitotoxic degeneration in vitro and in vivo, as well as against β-amyloid toxicity in vitro. The greater potency of MPEP with respect to SIB-1893 and SIB-1757 is consistent with its higher affinity for recombinant mGlu5 receptors. We cannot exclude the possibility that additional mechanisms are involved in the neuroprotective activity of MPEP, SIB-1757 and SIB-1893 because their EC_{50}

values in the neuroprotection assay did not correlate closely with their affinities at recombinantly expressed mGlu5 receptors. However, it is unlikely that neuroprotection results from a non-specific interaction of these hydrophobic compounds with the plasma membrane because iso-MPEP, a structural analogue of MPEP that did not interact with mGlu5 receptors, was inactive against NMDA toxicity. When tested in cultured cortical cells, SIB-1757 was less efficacious, although more potent, than SIB-1893 or MPEP as a neuroprotectant (about 60% of inhibition of NMDA toxicity, as compared with 80-90% of MPEP or SIB-1893). Since SIB-1757 and SIB-1893 have the same affinities for mGlu5 receptors, we conclude that the component antagonized by SIB-1757 represents the real contribution of endogenous mGlu5 receptors to the development of excitotoxic death. SIB-1893 and MPEP might share an additional component of neuroprotection, the identity of which is unknown at present. The unexpectedly high potency of MPEP in attenuating β-amyloid toxicity might be inherent to this particular paradigm of neuronal death. Accordingly, we have induced β-amyloid toxicity in the presence of a cocktail of ionotropic glutamate receptor antagonists. i.e., under conditions in which neuronal death follows an apoptotic pathway (Copani et al., 1995), whereas necrotic death is prevalent in response to an excitotoxic pulse with NMDA (Choi, 1992). Thus, one can speculate that endogenous activation of mGlu5 receptors contributes more efficiently to the development of an apoptotic programme rather than to the cascade of events leading to necrotic death, or, more simply, that the milder toxicity induced by β-amyloid is more sensitive to low concentrations of MPEP.

Taken collectively, these results suggest that, at least in the models we have used (see Nicoletti et al., 1999), activation of mGlu5 receptors by the glutamate endogenously released in the cultures contributes to neuronal degeneration in response to NMDA or β-amyloid peptide. Activation of postsynaptic mGlu5 receptors, by the endogenous glutamate, may contribute to the increase in cytosolic free Ca^{2+} through the formation of inositol-1,4,5-trisphosphate, as well as to the activation of NMDA receptors through the protein-kinase-C-dependent relief of the Mg^{2+} blockade of the NMDA channel (Chen and Huang, 1992). However, the latter mechanism cannot account for the protection of MPEP against β-amyloid toxicity, because experiments with β-amyloid peptide were always carried out in the presence of the NMDA receptor blocker, MK-801. Activation of presynaptic mGlu5 receptors (if any) by endogenous glutamate may also contribute to the development of excitotoxic death if one assumes that these receptors facilitate glutamate release. This, however, is a complicated issue because the influence on glutamate release depends on the functional status of group-I mGlu receptors (Herrero et al., 1998). It is intriguing that, both in cultured cortical neurons and in the intact striatum, selective mGlu1 receptor antagonists (such as LY367385, AIDA and CPCCOEt) are also substantially protective against NMDA toxicity (Bruno et al., 1999; Strasser et al., 1998) and β-amyloid toxicity (P. Flor, personal communication; but see also Allen et al., 1999 for contrasting data of AIDA on β-amyloid toxicity). Given the high degree of selectivity of these novel mGlu1 and mGlu5 antagonists, it can be concluded only that mGlu1 and mGlu5 receptors are involved in two sequential steps in the chain of neurotoxic events affecting individual neurons or they subserve independent functions which, however, contribute to the same extent to the overall neuronal toxicity. For example, activation of mGlu5 receptors might facilitate the opening of NMDA channels whereas activation of mGlu1 receptors might contribute to neurotoxicity by inhibiting GABA release (Pellegrini-Giampietro et al., 1999; Battaglia et al., 1999).

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